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A. INTRODUCTION

The biological condition, which led to the investigations on the thermal stability of the core protein of TMV mutants *in vitro*, is summarised in Part I. The primary structure of the protein, as determined by the protein-chemical investigations of Wittmann and coworkers (1962, 1964, 1965) and electrophoretic studies, are shown in Figure 1 along with data on the *in vivo* behavior (Part I).

B. MATERIALS AND METHODS

I. The isolation, propagation, and recovery of the virus mutants have been described in Part I.

II. Purification of Core Proteins

In the case of the previous *in vitro* studies (Jockusch, 1964), alkali-split virus was employed whose RNA was uniformly degraded by incubation with RNase. It was not, however, feasible to separate the RNA fragments completely from the protein by dialysis. For more exact studies, the preparation of RNA-free, native protein was necessary. The acetic acid method (Fraenkel-Conrat, 1957) yielded a very small amount of product since the protein must be renatured from the acetic acid solution. In the case of the alkaline cleavage of the virus, the senstive mutant protein remained completely native, but the separation of RNA and protein by ammonium sulfate (Schramm, 1947) gave unsatisfactory results. The electrophoretic separation of cleavage products (Schramm, 1947) offered the only practical means of obtaining high yields of labile protein. By means of carrier-free (?) electrophoresis ("Elphor VaP, Bender and Hobein, Munich) using the method of Hanning, a large amount of mutant protein cannbe

Abbreviations used: TMV=Tobacco Mosaic Virus; HP = core protein; RNA = ribonucleic acid; RNase = ribonuclease; tr = temperature-resistant; ts = temperature-sensitive; def = defect; AR = amino acid residue; Tris = 2-amino-2hydroxymethyl-propandiol -(1,3).

purified in a relatively short time (Sarkar, 1960). Moreover, the previously specified conditions (Jockusch, 1966) were adhered to. Al4 and its derivatives (except Ni511) split so poorly in alkali that the yield of protein and the purity achieved were similar to that with vulgare and its derivatives. For this reason, an "acetic acid protein" was employed in the case of Al4 and Ni696. No differences could be detected between the "alkali-protein" and the "acetic acid-protein" of Al4. Purity criterion: the degree of contamination by RNA was determined by means of the ultraviolet extinction coefficient E_{260}/E_{280} (corrected for light scattering). In the case of the complete virus (5 % RNA), a value of 1.2 was obtained. In the case of the "A-protein" of vulgare and its mutants, it was 0.55 to 0.58 indicating that the preparation was practically RNA-free. In the case of the Al4 mutants, a value of 0.60 to 0.64 was obtained. Native state activity: proteins were considered to be native if they produced clear solutions at 4°C and at pH 7, and if reversible opalescence was produced at pH 5 (except Ni2204 which reversibly precipitated at pH 5). Moreover, the proteins were preserved at pH 5, I (ionic strength)= 0.1 at -60°C.

III. Buffers Employed

In general, the buffer described by Miller and Golder (1950), which has an ionic strength of $\Gamma/2 = I = 0.1$, was employed for the denaturation studies. However, at pH 8, Tris was employed for buffering instead of veronal. The buffer has the following advantages: (a) the ionic strength is definite and is not affected by the pH, (b) 80 % of the ionic strength is the result of the NaCl present, (c) the ionic strength is equivalent to that of physiological saline, (d) the temperature coefficients of Miller-Golder buffers containing acetate (pH 5) and phosphate (pH 6 and 7) are small enought to be disregarded, (e) the buffers employed interfer with the determination of protein concentration

by the measurement of UV absorption or by the ninhydrin test. In this case, pure phosphate buffer (I=0.1) of Green is used. For testing solubility, because of its high buffering capacity as compared to Miller-Golder buffer, the acetate buffer, pH 5.0, of Boyd was employed (directions are found in Rauen, Biochemical Handbook, 1st edition, 1956).

IV. Gel Electrophoresis of Proteins

Proteins prepared according to Part B.II. were suspended in Miller-Golder buffer, pH 5, precipitated with methanol-ether as in Part I, section C.V., and then dissolved in 8 M urea, 20 % sucrose in Aronsson-Grönvall buffer. Since Ni2204 protein cannot be separated in this manner, it was precipitated by the addition of 2 M ammonium acetate, pH 4.8. In other cases, the acetic acid protein is obtained from the virus and, after removal of the RNA by centrifugation, the protein is precipitated isoelectrically by the addition of some water and 2 M ammonium sulfate. The precipitate is washed with methanol-ether and dissolved in urea buffer. The protein concentration is about 1 to 5 mg per ml. Electrophoresis was carried out as described in Part I, Section B.IV.5.

V. Tests for Native State of Proteins

Next to the macroscopic examination of protein solutions (clear, opalescent, precipitate), the following criteria were used for determining the native state: solubility at pH 5, development of soluble particles with sedimentation coefficients of greater than 100S, which appear as aggregates when observed under the electron microscope with a diameter of about 150 Å and a central canal.

1. Ultracentrifugation.

This was carried out using a Spinco Model E analytical ultracentrifuge (Beckman Instruments) with an An-D rotor. In order to avoid strong shearing forces upon injection of the sample, a covered cell was used into which the sample could be pipetted. It was not, therefore, layered with buffer (with the

exception of the urea protein), but was filled with 0.7 ml of protein solution in order to create an adequate sedimentation path. All runs were carried out at 20°C. The sedimentation coefficient S was calculated as follows:

$$\text{Log } S = 13{,}1818 + \text{Log}\Delta \text{Ln } r - (2\text{Log } f + \text{Log}\Delta t)$$

where S (S)

r (cm) the radial distance of the gradient from the axis.

$\Delta \text{Ln } r = \text{Ln } r(t_2) - \text{Ln } r(t_1)$

Δt (min) $= t_2 - t_1$, the time period between photographs and

f (min^{-1}) the speed of rotation of the rotor

The viscosity was measured at 20°C using an Ostwald viscosimeter.

2. Electron Microscopy.

Protein aggregates were analyzed by the negative staining method of Brenner and Hormer (1959). A drop of the sample was applied with a capillary to the grid, placed under a vacuum and dried. The same procedure was repeated with 2 % phosphotungstic acid at pH 5. The electron micrographs were kindly prepared by Dr. H. Frank and Mr. Berger (Max Planck Institute for Virus Research) using a Siemens Electron Microscope I.

C. RESULTS

I. Electrophoretic Behavior of Proteins in 8 M Urea

Sengbusch (1965) found that intact virus particles of some mutants, where protein analyses (Wittmann, 1962) showed no differences between them and the parent strain, possessed a slight mobility in the electrical field in weakly alkaline solutions at intermediate ionic strength. This is explained by amino acid substitutions of the type: aminodicarboxylic acid → aminodicarboxylic acid amide. These are not normally detected by protein analysis. RNA-free proteins in 8 M urea were analyzed by gel electrophoresis in order to exclude the

possibility that substitutions, in addition to those indicated by Wittmann, may occur, and to characterize several substitutions where it is not known whether the aminodicarboxylic acid or its amide is present (Wittmann, 1962). Figure 2 shows the bands that were obtained. Two classes of bands are seen: some, which migrate like vulgare, and some which migrate like flavum. This difference is based upon a single charge difference (Wittmann et al., 1965). By some derrangement during aggregation (secondary bands) or carbamidisation of amino groups by cyanate (Cole and Mecham, 1965; in alkaline medium), the results were in agreement with the analyses of Wittmann as well as the electrophoretic studies of Sengbusch. Earlier, it was shown that in the case of Ni103 and Ni696, an aspartic acid residue is substituted in position 19 of the polypeptide chain and that the single aspartic acid is in peptide I. Ni109 does not migrate at a slower rate than vulgare as one would expect from the protein - chemical results (Wittmann, 1962) and the findings of Sengbusch (1965). There are two possible explanations for these results: (a) the protein contains an additional substitution of aminocarboxylic acid → aminodicarboxylic acid which is not detectable by protein analytical methods. This additional substitution neutralizes the charge loss caused by the glutamic acid → glycine substitution in the case of urea-extracted protein. (b) We are dealing with nothing more than the original parent strain. In any case, it is out of the question that the protein contains no substitution different from vulgare.

If one begins with only the results obtained with electrophoresis, the following principle is formulated: All proteins, which migrate more slowly than the vulgare protein in alkaline solvents, are ts (temperature-sensitive). However, not all ts-proteins migrate more slowly than vulgare protein. The mutants, Ni118 and Ni1196, which have both lost a proline by mutation, form only weak and diffuse bands. In alkaline 8 M urea solutions, they exhibit more aggreagtes

than residual protein. In this regard, previous investigations with the analytical centrifuge have shown that Ni118 protein in this solvent sediments about twice as fast as does vulgare protein.

II. Pattern Tests with Ni118 and Vulgare Proteins

Ni118 was selected for these tests since it is a sensitive ts mutant and its protein exhibits no charge substitution as compared to the vulgare protein. This was done so that the effects that were observed would not be dependent on different isoelectric points.

1. Speed of Denaturation.

For the following studies, the protein, which was at pH 5, I=0.1, was dialyzed for at least 48 hours at 4°C against repeated changes of buffer. The protein solution was then incubated in a water bath under the actual experimental conditions. At specified times, 0.5 ml of protein solution was withdrawn and pipetted into 1.5 ml of ice cold Boyd buffer, pH 5. After thorough mixing, the sample was allowed to set for several minutes in an ice bath and then centrifuged for 4 minutes at 1,300 x g (solubility test). Denatured protein under these conditions will be found in the sediment after centrifugation. The protein content of the supernatant fractions was determined after alkaline hydrolysis using the ninhydrin reagent according to Moore and Stein (1954). The protein concentration for this experiment was calculated from the following relationship: 1.10 $O.D._{280\,m\mu}^{1\,cm}$/ml $\hat{=}$ 5×10^{-5} M (Fraenkel-Conrat, 1957).

a) Effect of Temperature. Figure 3 shows the denaturation of Ni118 protein at 20°C and 30°C. The samples contained 5×10^{-5} M protein in Miller-Golder buffer, pH 7.0, I=0.1, 10^{-3} M ethylmercaptan. The capacity to form soluble aggregates at pH 5 disappears at 30°C with a half time of $t_{\frac{1}{2}}$=+1.8. At 20°C, $t_{\frac{1}{2}} > 10^2$ minutes. The "melting point" of the tertiary structure of Ni118 protein (Kauzmann, 1954) as a consequence of an amino acid substitution lies

between 20° and 30°C, while it is greater than 30°C in the case of the vulgare protein. However, at 20°C, appreciable denaturation occurs after 5 hours (Table 1).

b) Effect of Mercaptan. Duplicate samples with 5×10^{-5} M Ni118 protein in Miller-Golder buffer, pH 7.0, I = 0.1, were incubated at 30°C with and without 5×10^{-3} M ethylmercaptan. In order to assimilate the test conditions, the Boyd buffer contained 10^{-2} M ethylmercaptan. Even with a 100-fold increase in SH-groups over those in the protein, no differences in the speed of denaturation during incubation could be found (Figure 4). Nevertheless, the studies were carried out in the presence of mercaptan in order to avoid any troublesome interferences by heavy metal contamination.

2. Aggregation during Dialysis.

If, during the investigations on the progress of denaturation, the pH is lowered to 5, optimal aggregation to rods is achieved in this case as well as by dialysis. In the following studies, the behavior of Ni118 protein and vulgare protein was studied at 20° and 30°C at pH 7.0 or by dialysis at pH 5. These were carried out using 10^{-4} solutions of Ni118 and vulgare protein. All of the incubation buffers contained 10^{-2} M ethylmercaptan. The dialysis was against Miller-Golder buffer at pH 5.0. The incubation and dialysis times for all samples were 5 hours, after which the samples were centrifuged for 10 minutes at 1300 g. The relative concentrations of the sediments and supernatants were determined by measurements of the E_{280} after evaporation and dissolution in 10 M urea, pH 7. The supernatants were centrifuged in the analytical ultracentrifuge and the soluble fractions were studied under the electron microscope. Table 1 gives the fractions of the supernatant and precipitate and the sedimentation coefficients of the proteins in the supernatants. Figure 5 shows the sedimentation profile, while Figure 6 shows the electron micrographs of aggregates found at pH 5. The results are (a) by dialysis pH 7 → 5 at 20° and 30°C,

vulgare protein forms aggregates of greater than 100 S in which, as the electron microscope controls show, the subunits are arranged in a hollow cylinder form. The fraction of protein that precipitates in this case is less than 1 %. At pH 7 and I = 0.1, the protein remains in a clear solution in the form of smaller aggregates of ca. 30 S. This[1] corresponds to a double ring of about 2 x 17 protein subunits.(Caspar, 1963). At I = 0.02, and after storage at 4°C, a component with a value of 4 S was found. This corresponds to the actual "A protein" with three subunits per particle (Caspar, 1963). (b) Like vulgare, Ni118 protein after dialysis pH 7 → 5 at 20°C, forms soluble aggregates of more than 100 S which appear as rods (hollow cylinders) under the electron microscope. About 99 % of the protein in this case forms into aggregates. However, at 30°C and I = 0.1, this value is 75 %, and at I = 0.02, 98 % of the protein forms an insoluble precipitate. The somewhat higher stability at higher ionic strengths can be shown to be reproducible. At pH 7 → 5, I =0.1, the vast majority of the protein in solution is inthe form of rods. Hence, it has been concluded that the 30°C-denaturation of Ni118 protein is an all-or-none occurrence, that is, either the protein precipitates or rods are formed at pH 5. At pH 7, I = 0.1, after 5 hours at 20°C, a slight precipitate is formed. At 30°C, 91 % of the protein precipitates. At I = 0.02, however, the solution remains clear and particles are found with a sedimentation coefficient of 36 S.

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1 The sedimentation coefficient values are not corrected to a constant viscosity. Viscosity measurements were carried out on A proteins at pH 7, aggregates at pH 5, and virus particles at pH 7. With a protein concentration of 10^{-4} M, the viscosity of the aggregates was only 6 % higher than that of the pure solution, It is concluded that it would not be important to correct for such a small change.

(c) The question now was if the 36 S component obtained by incubating H118 protein at 30°C, pH 7, I = 0.02, still possessed the capacity to form aggregates in an orderly fashion when dialysed against pH 5 buffer in the cold. It was found that after incubation for 30 minutes at 30°C followed by dialysis at 4°C against Miller-Golder buffer, pH 5,0, I = 0.1, 95 % of the protein precipitated. Contrary to an earlier assumption (Jockusch, 1964), the protein remaining in solution at 30°C at low ionic strength is no longer native. On the other hand, vulgare protein can be kept under similar conditions for at least 24 hours at 30°C without losing its capacity to form soluble aggregates at pH 5 in the cold.

(d) Stabilisation by phosphate ions: The precipitation of H118 protein at 30°C in Miller-Golder buffer, pH 7, I = 0.1, is not purely an ionic strength effect. If one incubates the protein under similar conditions but in pure phosphate buffer, pH 7, I = 0.1, the solution will be only opalescent and not removed by centrifugation at 1,300 x g. After dialysis at 4°C against buffer, pH 5, I = 0.1, only a fraction of the protein precipitates. In contrast to chloride ions, the phosphate ion has a stabilizing effect at least in the case of the solubility behavior of the H118 protein.

3. Nature of the Precipitates.

From the spectrum of the solvents which are capable of dissolving the protein precipitates, one can draw some conclusion as to the nature of the bonds involved in holding the aggregate together (Summary: Jaenicke, 1965). Using H118 protein, the following studies were carried out: A 10^{-4} M solution of native protein in buffer, pH 7, I = 0.1 with 10^{-3} M cysteine, was held at 30° C for 40 minutes and then mixed with an equal volume of Boyd buffer, pH 5, I = 0.1 (the resulting pH was at 5). The suspension was divided up and the fractions were centrifuged at 1,300 x g for 10 minutes. 21 % of the protein was recovered in the supernatant fractions. The sediments were suspended in equal volumes of various

solvents as shown in Table 2. The protein concentration in the supernatants was determined on the basis of the E_{280} (Table 2) after centrifugation for 10 minutes at 1,300 x g. It was found that (a) the coherence of the aggregates is not the result of covalent binding. Mutual exchanges between aliphatic side chains appear to be the major contribution (solubility in sodium dodecylsulfate). (b) The binding forces differ qualitatively from those which hold the native protein aggregate together (higher alkali resistance). (c) At pH 5, at 4°C as well as at 30°C, a noteworthy renaturation occurs.

III. Comparative Denaturation Kinetics of Vulgare Mutants

1. Denaturation at pH 7.

The four tr strains, vulgare, A14, N1109, N11927, and the five ts strains, flavum, reflavescens, CP415, N1103, and N1118, were compared at pH 7, I = 0.1, 30°C, with reference to the velocity of protein denaturation (Figure 7). These were tested as described in Section C.II.1. With the exception of reflavescens, which is derived from flavum by way of the decens strain, all of the mutants differ from vulgare by only a single amino acid substitution (with regards to N1109, however, see Section C.I.). With regards to their ability to form soluble aggregates at pH 5, in the case of the ts-mutants, half-lives of 1.8 minutes (N1118) to 80 minutes (reflavescens) were observed. N11927 was more strongly denatured than vulgare whose half life was >2.5 x 10^3 minutes which was greater than that of the most stable ts-mutant. The protein from A14, however, is even more stable. After 15 hours, practically no denaturation can be detected using the solubility test in the case of the vulgare protein.

2. Effect of PH

Multiple samples were suspended in buffers of pH 8.0, 6.0, and 5.0 as described in Section C.III.1., incubated, and test. Figure 8 shows the corresponding curves for pH 6.0; at pH 5.0, after 15 hours, practically no

decrease in solubility was detected. At pH 8, the proteins behaved the same as at pH 7.

The half-lives obtained in this manner are summarized in Table 3. As the incubation pH approached the isoelectric point, the denaturation velocities were decreased by a factor of >100 to >1000 (Ni118).

IV. Comparison of Several A14 Mutants

In Figure 9 is shown the kinetics of the loss of solubility of several A14 mutant proteins incubated at pH 7, I=0.1, as described in Section C.II.1. The half-lives found were: Ni511 - 20 minutes; Ni696 - 30 minutes; Ni1196 - 180 minutes; Ni2519 - $>2.5 \times 10^2$ minutes.

One finds that (a) the mutant Ni696, which is differentiated from flavum by two very distinct substitutions in the polypeptide chain, is somewhat more stable than flavum: Ni696 $t_{\frac{1}{2}}$ = 30 minutes; flavum $t_{\frac{1}{2}}$ = 10 minutes. (b) Also, an amide substitution can cause ts behavior (see C.I.). The protein of Ni511 has a similar half life to that of flavum. (c) The protein of the ts mutant Ni2519 is as stable as that of A14 at 30°C. Also, no precipitation was observed during dialysis of alkaline-split virus against pH 5 buffer at 35°C. In aggrement with the protein analyses of Wittmann-Liebold et al. (1965), in both mutants, no substitution could be found and from these and the in vivo studies (Part I), one must conclude that in the case of Ni2519, it is not the envelope protein that is altered but rather there is the mutation of a second gene for temperature sensitivity. (d) Ni2204 precipitates independently from the 30°C influence almost completely at pH 5, I = 0.1.

V. Wild Strains

In the case of the in vivo studies (Part I), it was shown that the wild strains, dahlemense, U2, and Holmes' Rib Grass, are tr. In the case of the

in vitro studies, the test conditions for U2 and HRG in contrast to those for *vulgare* must be somewhat altered since isoelectric precipitation occurs at a higher pH. It was diluted with Boyd buffer, pH 5.5, I = 0.1, insteand of pH 5 (see Rentschler, in preparation). Incubation at pH 9.0, I = 0.1, 10^{-3} M ethylmercaptan, 30°C for 4 hours, gave the following results: U2 and HRG are completely stable; *dahlemense* protein denatures very slowly: $t_{\frac{1}{2}} \approx 300$ minutes.

VI. In Vitro Stability of Virus Particles

From the fact that during virus recovery, heat precipitation of plant proteins at 60°C for 10 minutes is utilized and that this method allows the recovery of both *tr* and *ts* mutants, it has been tentatively assumed that complete *ts* virus particles have the same high thermal stability as the *vulgare* virus (Jockusch, 1964). In making this assumption, however, it is necessary to consider the following: (a) The conditions in the crude plant extracts are not defined, particularly with regards to the actual initial virus concentration, which is not known. (b) The treatment time is relatively short as pppoesd to the time of *in vivo* growth (several days). (c) Particularly in the case of the *ts* mutants, one often obtains unpredictable and often low virus yields which could well be the consequence of the heat treatment. In the following experiment, it was determined if incubation of *ts* virus particles for long periods of time at high temperatures rendered them RNase sensitive. Solutions of purified virus containing 1 mg/ml of strains *vulgare* (*tr*), A14 (*tr*), Ni118 (*ts*) *flavum* (*ts*), CP415 (*ts*) were incubated in Sorensen phosphate buffer, pH 7.0, I=0.17, at 50°C. at times 0, 5, 15, 25, and 40 hours, samples were withdrawn, mixed with an equal volume of cold solution containing 10 µg/ml RNase[2] in phosphate buffer, pH 7, and then incubated for 1 hour at 37°C. After that, the samples were diluted in ice cold phosphate buffer (1:1000 for *vulgare*, A14, and Ni118; 1:200 for *flavum* and Cp415)

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[2] Worthington Biochemicals, Freehold, N.J. U.S.A.

and held in an ice bath until the test. Infectivity was tested on the Xanthi tobacco strain. Control: untreated vulgare solutions containing 1.0, 0.2 and 0.02 µg/ml virus. Vulgare solutions were incubated for 40 hours at 50°C without subsequent RNase treatment. Vulgare RNA, 20 µg/ml, untreated as well as virus solution treated with RNase were diluted to 5 µg/ml for the test. The results are shown in Figure 10.

None of the strains showed a significant decrease in infectivity. As the dilution series for the vulgare virus shows, it doesn't matter if the test was carried out in the saturation range of the standard curve. After treatment at 50°C for 40 hours, vulgare virus not treated with RNase showed no significant difference in infectivity as compared to virus treated with RNase. Under the same conditions, treatment of free RNA with RNase was 100 % effective in inactivation.

D. CONCLUSIONS

I. Relationship of In Vivo and In Vitro Behavior

The known functions of the TMV envelope protein are (a) in the cytoplasm of the host plant, they aggregate specifically with virus RNA in forming virus rods in the presence of other proteins and nucleic acids. (B) During the extracellular phase of the infection cycle, they provide the RNA encasediin the viable virus rod with adequate protection against external factors, particlarly the action of RNase. (c) In spite of this stability, they permit the release of RNA during the primary process of infection. The physicochemical basis is known for for only one of the functions. Function (b) is not, according to Section C.VI., temperature sensitive in the case of the ts-mutants. The following results show that the aggregation of RNA-free envelope proteins at low pH is an appropriate model for the coaggregation of the proteins with RNA: (a) The defective mutant, PM2, does not form stable virus particles in the plant under green house conditions

(Siegel, Zaitlin, and Sehgal, 1962). In vitro, at low temperatures, its proteins aggregate in such a way so as to form open helices which do not have the conformation necessary to protect RNA from RNase (Zaitlin and Ferris, 1964). (b) The mutant Ni2204, at high as well as at low temperatures, constructs very small amounts of stable virus - its protein precipitates also in the cold upon dialysis against buffer, pH 5.0, I = 0.1, whereas the remaining mutants form rod-shaped aggregates. (c) Ts mutants of Class I, in contrast to the wild type vulgare and the tr mutants, can form only very reduced amounts of virus at 32°C in vitro - Their proteins lose up to 50 % of the capability to form orderly aggregates in pH 5 buffer after incubation at 30°C, pH 7, I=0.1, in 2 to 130 minutes. In contrast, the capabilities of the tr proteins in this respect remains undiminished for many hours. Obviously, in this case, low pH can alter the phosphate residues of RNA during the aggregation process if the fine structure of the rod-shaped aggregates ("stacked disk" form) is not in close enough harmony with that of the virus, (Macrohelix)(Franklin and Commoner, 1955). Considered collectively, the in vivo and in vitro investigations hshow the unequivocal conclusion that temperature sensitivity of mutants of Class I is dependent on the temperature sensitivity of the envelope protein subunits.

II. The Aggregation Defect as A Consequence of The Irreversible Alteration of Conformation

In the case of the first in vitro studies on ts mutants of TMV (Jockusch, 1964), aggregation and heat treatment were carried out simultaneously (dialysis at high to low pH at 30°C). It was presumed consequently that high temperature during the aggregation process produced denaturation. The newer in vitro studies (this investigation and Jockusch, 1960) showed, however, that the denaturation of envelope protein subunits occurs also at constant high pH and

that the disordered aggregation during the subsequent pH reduction is a consequence of this denaturation.

III. Stabilization by Low pH or RNA

The reason of denaturation of proteins by heat is the thermal movement of the polypeptide chain components away from the configuration characteristic of the native molecule, which leads to the formation of stronger bonds of the polypeptide components of the same molecule in a different manner or with another molecule (Wu, 1931). From the structure of the protein aggregate and the complete virus particle, one is able to comprehend how the degrees of freedom for thermal movement by the polypeptide chain is reduced as a result of the small distance between subunits in the A-protein. Consequently, it is plausible to assume that low pH as well as RNA produce thermal stabilization of the subunits during aggregation. In this regard, it can be also said that the pH dependence of the denaturation speed at pH $\geq$ 7 is practically nonexistent.

IV. The Importance of A Single Amino Acid Residue

As is a result of the protein-chemical analyses of Wittmann and co-workers, it is possible to correlate the in vivo and in vitro behavior of mutant proteins not only with each other but also with the known amino acid substitutions. In this case, however, a simple, generalized rule cannot be established (Wittmann-Liebold et al., 1965; Jockusch, 1966). For that reason, only a few specific cases can chosen where a plausible explanation can now be given.

1. The Proline Residue in Positions 20, 63, and 156

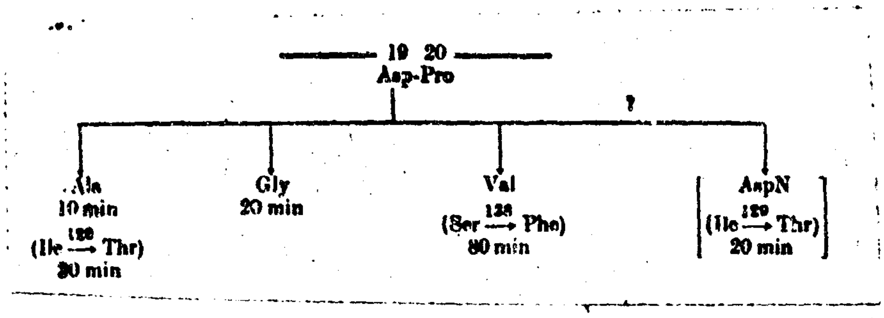
Ni 118 (Proline → leucine in position 20) and Ni1927 (proline → leucine in position 156) constitute a very clear example of the effect of the position of the substitution in the polypeptide chain on the stability of mutant proteins (Jockusch, 1964; Wittmann-Liebold et al., 1965). Ni1927 is tr. The C-terminal

end (position 158) of the polypeptide chain lies on the surface of the virus particle (Harris and Knight, 1952) and, as a consequence, also on the surface of each subunit. Ni118 is ts. The substitution at position 20 is not detectable serologically in the intact virus and appears, therefore, at least from the observations with the whole virus particle, to lie more towards the interior (Sengbusch, 1965). Obviously, the proline at position 20 stablizes a sharp bend in the polypeptide chain which is necessary for the functioning of the subunit (orderly aggregation). The proline residue, however, is not important in the spontaneous production of these sharp bends at higher temperatures. In contrast to this, the three dimensional fixation of either of these amino acid residues by the proline residue in position 156 does not appear to be decisive in the reaggregation and the back-folding of the proteins denatured with urea: hence, it follows that the last three amino acids can be generally absent (Sengbusch and Wittmann, 1965). The temperature sensitivity of Ni1196 (proline → serine in position 63) can be understood as well as that of Ni118 but it is not possible at this time to give an explanation for the quantitative differences between these ts mutants.

2. The Groups in Positions 19 and 20.

The group, aspartic acid - proline, which is found in vulgare, is also found in hemoglobin and causes, in the latter case, the break in the alpha-helix region (Perutz et al., 1966: Guzzo, 1966). The end of the helical region is quite generally an important prerequisite for formation of the structure of globular proteins. In the group 19-20 in the case of TMV envelope proteins, either proline or aspartic acid can be substituted. In both cases at 20-25°C, the functional tertiary structure will be improved but the disaggregated protein will be labile at high temperature. The aspartic acid has been

substituted by various amino acid residues in different mutants:



(other amino acid substitutions are shown in parenthesis)

All of the substituted amino acid residues result in the appearance of <u>ts</u> protein. However, the native state half-lives measured at 30°C, pH 7, I = 0.1, differeas shown above. The fact that the substitution, amino dicarboxylic acid ⟶ amino dicarboxylic acid amine is aspartic acid ⟶ asparagine at position 19 in Ni511 is a speculation based on physicochemical similarities between these proteins and those of flavum, Ni103, and Ni696. In must remain undecided, however, whether the stabilizing effect of aspartic acid is the result of a mutual exchange with the solvent (Guzz0, 1966) or the result on an intramolecular salt bond.

<u>3. The Wild Strain Protein</u>

The four wild strains, <u>vulgare</u>, <u>dahlemense</u>, U2 and Holmes' Rib Grass (HRG) are <u>tr in vivo</u> and <u>in vitro</u>. Obviously, they have been selected under natural conditions for temperature resistance. The proteins from <u>dahlemense</u> (Wittmann-Liebold and Wittmann, 1963) and U2 (Wittmann, 1965; Rentschler, in press) have been analyzed for their primary structure. The protein-chemical basis of their temperature resistance in shown in Figure 1: Positions, where mutations can lead to temperature sensitivity are similar to those of <u>vulgare</u> in the case of the other wild strains. However, those positions which do not affect <u>tr</u> behavior vary widely (Ni116 and Ni2068 belong to the stable <u>ts</u> mutants. The proline residue in position 156 is involved with enzymatic stabilization (Tsugita and Fraenkel-Conrat, 1960)).

4. The "Lipophilic" Protein of Ni2204

The protein of Ni2204 differs from all the other mutant proteins studied in that (a) it is reversibly precipitated at pH 5, I = 0.1, 4°C, and (b) it cannot be precipitated from an aqueous solution with methanol-ether. Its strong lipophilic behavior can be explained by the substitutions: serine ⟶ leucine (position 15) and threonine ⟶ isoleucine (position 153) if one assumes that they lie in the vicinity of the surface of the subunit. Both of the substitutions are of the type: polar   apolar (see Figure 1).

SUMMARY

The proteins of several temperature sensitive ~~(ts)~~ and temperature-resistant ~~(tr)~~ strains of TMV ~~(comp. Part I)~~, for which the amino acid sequence is known due to the work of Wittmann et al., ~~have been~~ separated from the RNA in the native state and investigated with respect to their thermal stability.

Model experiments with the wild type (vulgare) and the most sensitive mutant (Ni118) show that:

(a) Upon dialysis from pH 7 to 5 at 30°C, Ni118 protein forms a precipitate and only a small proportion of soluble aggregates, whereas Ni118 protein at 20°C and vulgare protein at both temperatures form soluble aggregates with sedimentation coefficients of several hundred S (Fig. 5, Table I).

(b) If soluble aggregates are formed, they have the typical cylindrical structure (Figure 6).

(c) The non-covalent bonds which form the precipitate differ qualitatively from those which form the ordered aggregates (Table 2).

The thermal stabilities of 18 different proteins were compared. Upon incubation at pH 7, I = 0.1, 30°C, ts-I mutantsproteins lose their ability

to form soluble aggregates at pH 5 in the cold with half lives of 2 to 60 minutes (in one case 180 minutes), whereas the proteins of <u>tr</u> mutants retain this ability for at least several hours (Fig. 7, 9). Of the wild type proteins, <u>dahlemense</u> is the most sensitive. In contrast to all other mutant proteins, the protein of the defective mutant Ni2204 precipitates at pH 5 even in the cold. The protein of the <u>ts</u>-II mutant Ni2519 is stable at 30°C (Figure 9).

The rate of denaturation of <u>ts</u> proteins decreases strikingly when the pH is lowered from 7 to 5, but between pH 7 and 8, there is only a slight pH dependence (Figure 8, Table 3). Complete virus particles of <u>ts</u> mutants are stable at 50°C for at least 40 hours (Figure 10).

The role of certain amino acid residues in the stabilisation of the native tertiary structure is discussed. In the case of <u>ts</u> mutants, all the amino acid residues which are replaced are not necessary for the spontaneous formation of a functional structure at low temperature.

It was found that:

(a) All mutant proteins which have a lowered electrophoretic mobility in 8 M urea at pH 8.8 are <u>ts</u>, but not all <u>ts</u> proteins have an altered electrophoretic mobility.

(b) The proline residues in positions 20 and 63 are necessary for the thermal stabilisation of disaggregated protein, but not the proline residue in position 156.

(c) In the positions 19-20, the arrangement, Asp-Pro, most probably stabilises a bend in the polypeptide chain which is necessary for ordered aggregation. The substitution of each of the residues leads to a sensitive protein.

(d) In the case of the defective protein of Ni2204, the role of polar (but not charged) and apolar side chains for the solubility properties of the protein molecule is evident.

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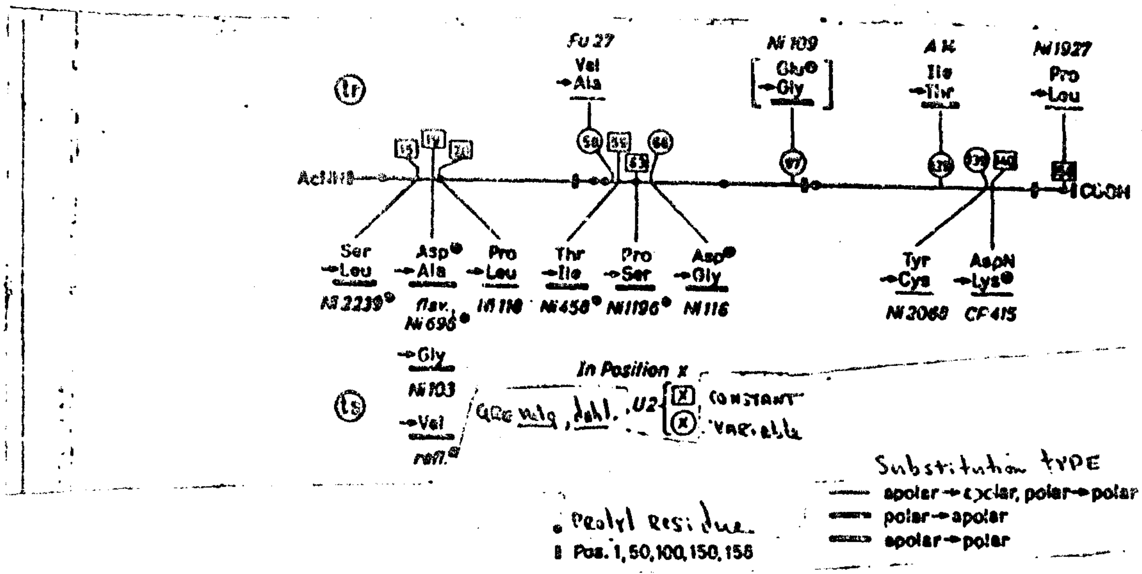


Fig. 1. The primary structures of the envelope proteins and the in vivo temperature behavior of 14 mutants of TMV. Not shown are: Ni2204* (def): serine → leucine in position 15, threonine → isoleucine in position 153. * In addition to the substitutions indicated, in A14 there is present the substitution isoleucine → threonine at a position which in itself does not lead to temperature sensitivity. [a] In addition to this substitution, there is the substitution serine → phenylalanine at position 138, which does not lead to temperature sensitivity according to studies on another mutant. The protein-chemical data have been obtained from Wittmann, 1964 and Wittmann-Liebold et al., 1965, for the mutants, and from Wittmann-Liebold and Wittmann, 1963; Wittmann, 1965, and Rentschler, in press, for the wild strains. Localization of substitutions of Ni103 and Ni696 within peptide I by electrophoresis - separation into tr and ts according to Jockusch, 1964, and this work, Part I - separation of amino acid residues as polar and apolar according to Perutz et al. (1965).

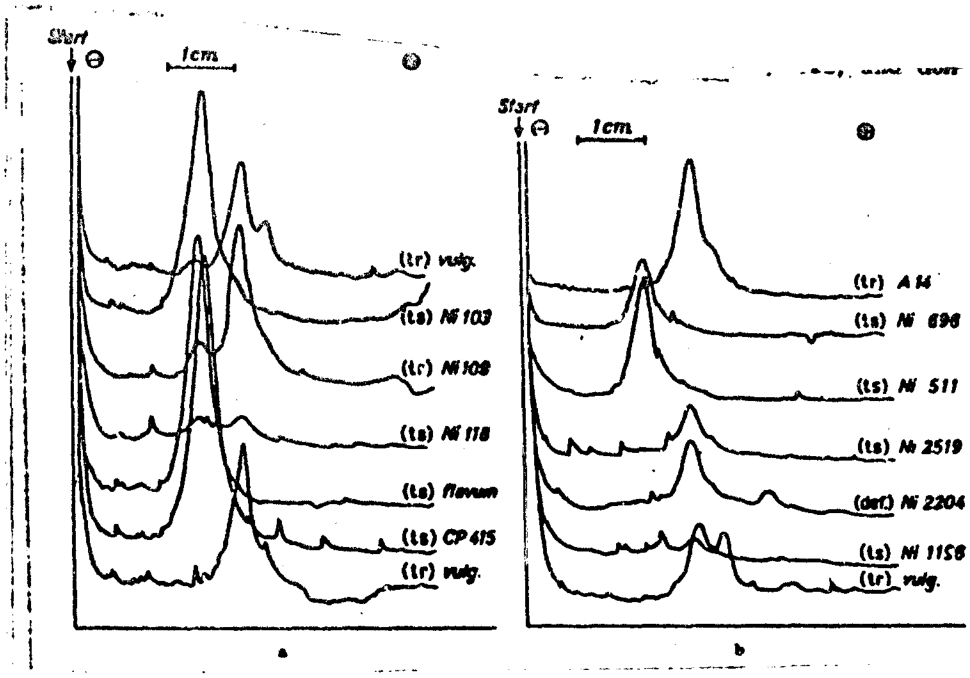


Fig. 2a and 2b. Gel electrophoresis of TMV mutant proteins in 8 M urea, pH 8. (a) Vulgare derived mutants, (b) A14 derived mutants. Reference: vulgare protein, 200 V, 48 --- 30 ma, 15 hours, dessitcmetry after amido-Schwarz staining.

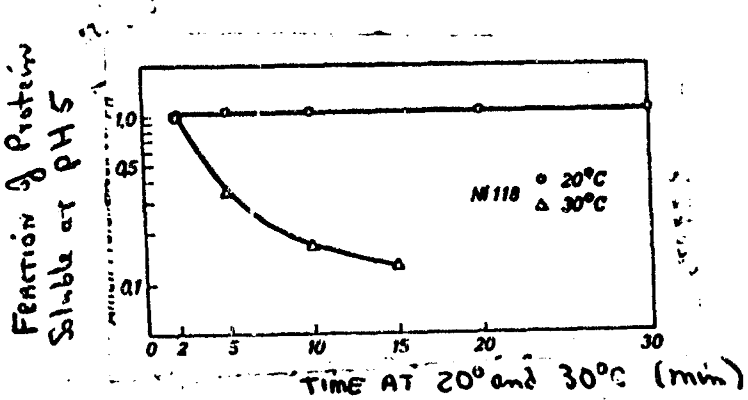


Fig. 3. Ni118 protein at pH 7.0, I ≑ 0.1, at 30°C and 20°C. Loss of capacity at pH 5 to form soluble aggregates.

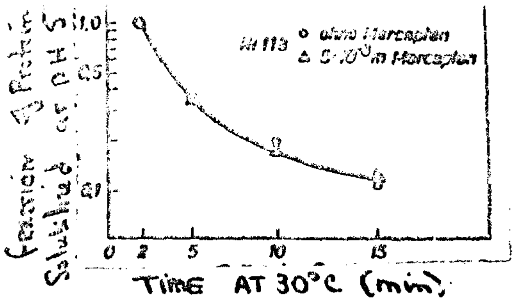


Figure 4. Denaturation kinetics of Ni118 protein at pH 7.0, I = 0.1, 30°C in the presence and absence of ethylmercaptan.

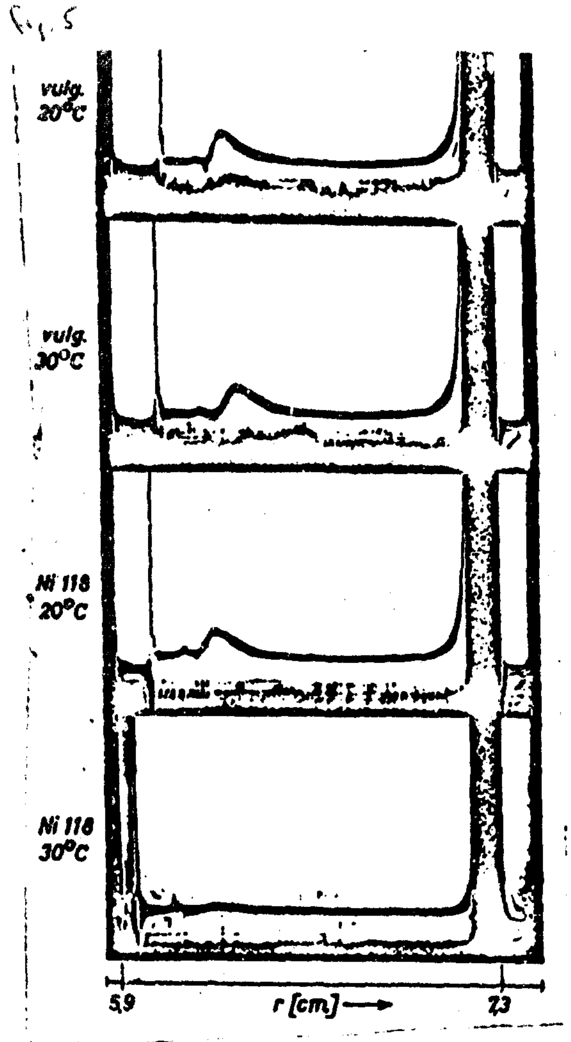


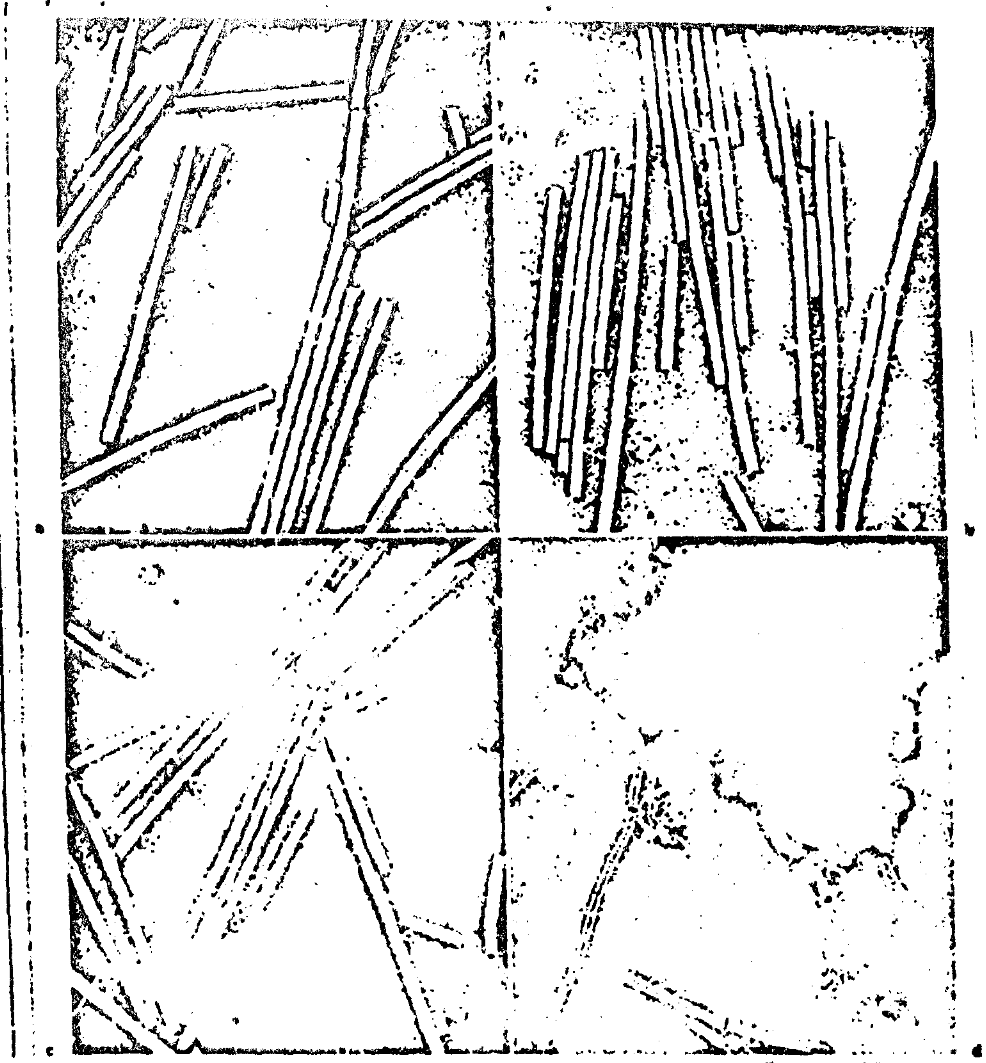
Figure 5. Sedimentation profiles of vulgare and Ni118 protein which have been dialyzed for five hours at $\overline{I = 0.1}$, from pH 7.0 to pH 5.0. Prior to analytical ultracentrifugation, the precipitated protein was

legend continued on next page

Legend for Figure 5, continued:

removed by centrifugation for 10 minutes at 1,300 g. Pictures taken ca. 2,5 minutes after attaining running speed of f = 17,980 (min^{-1}) with Philpot-Svensson optics, 60° angle.

Fig. 6



Figures 6a-d. Electron micrographs of protein aggregates of <u>vulgare</u> and Ni118. The results are similar to those in Figure 5 except that the solutions were not preparatively centrifuged. Undiluted aggregates were negatively stained with phosphotungstic acid. Apparatus magnification: 40,000 X; total magnification 100,000 X. <u>Vulgare</u> proteins: (a) at 20°C; (b) aggregated at 30°C. Ni118 protein: (c) at 20°C; (d) aggregated at 30°C. In (d), in addition to cylinderform aggregates, a number of amorphous aggregates are seen. 70 - 80 % of the protein is found in the amorphous aggregates. Pictures taken by Dr. Frank, Max Planck Institute for Virus Research.

Fig 7 + 8

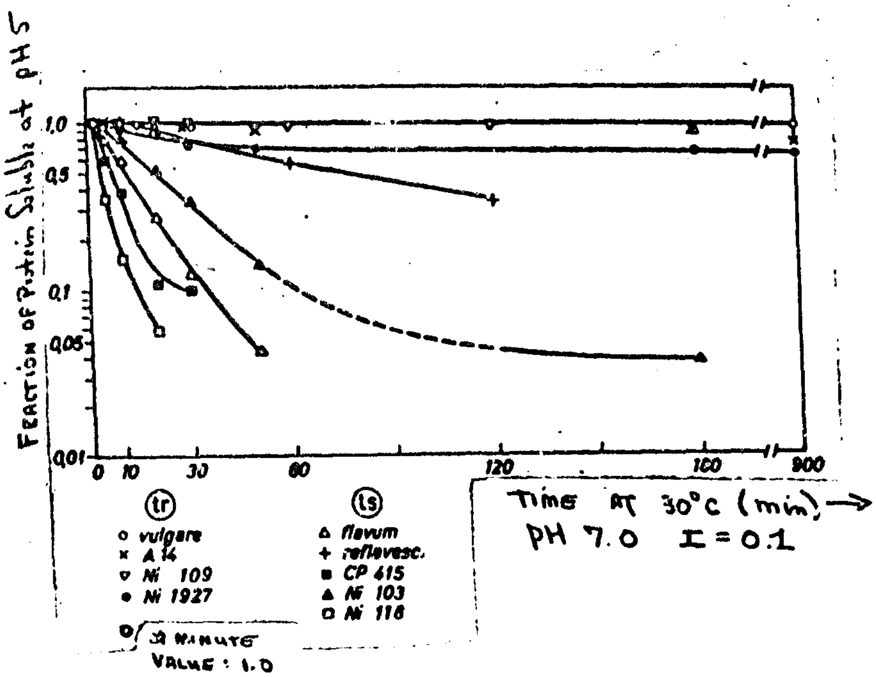


Figure 7. Denaturation kinetics of proteins from vulgaree mutants and reflavescens.

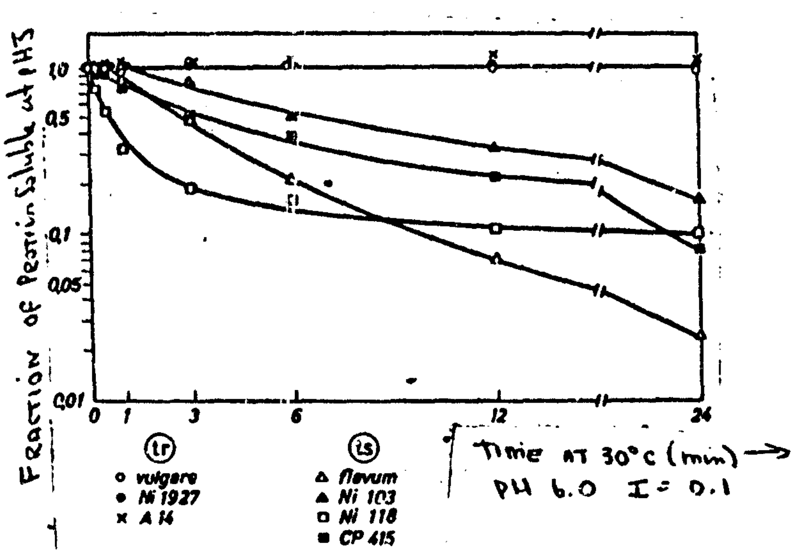


Figure 8. Denaturation of proteins from vulgare mutants at pH 6.0. Scale for the time axis differs from that in Figure 7.

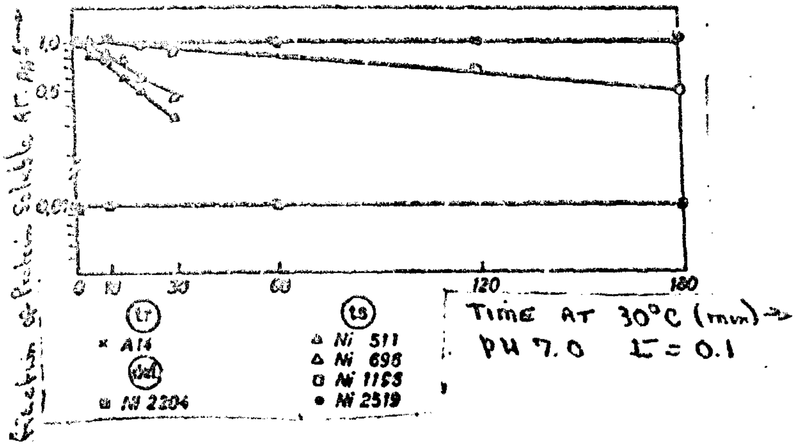


Figure 9. Denaturation kinetics of A-14 Mutant proteins.

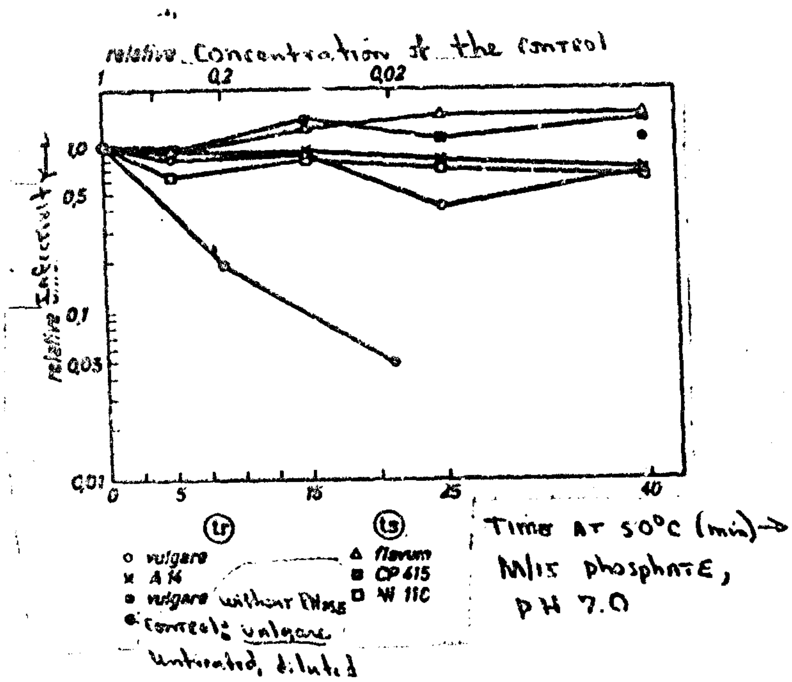


Figure 10. Thermal stability of tr and ts virus particles. See text.

TABLE I

Sedimentation Characteristics of Vulgare and Ni118 Proteins

		I = 0,1		I = 0,02	
		pH 7	pH 7→5	pH 7	pH 7→5
Ni 118	4° C	32 S	—	3—4 S * 30 S	—
	20° C	89% 30 S 11% Pr	99,5% 590 S (250 S) 0,5% Pr	—	—
	30° C	9% 15 S 91% Pr	26% 270 S 74% Pr	99% 36 S 1% Pr	2% 250 S 98% Pr
vulgare	4° C	—	—	4 S *)	—
	20° C	100% 29 S 0% Pr	99,5% 560 S (250 S) 0,5% Pr	—	—
	30° C	100% 26 S 0% Pr	99,5% 580 S (320 S) 0,5% Pr	—	99% {240 S, 530 S} 1% Pr

Similar studies are shown in Fig. 5. Given are the relative fractions in % of protein with the stated seddimentation characteristics (sedimentation coefficient in Svedberg units, in brackets, the next component, or "Pr" = precipitated). Determination of concentration by measurement of the E_{280} in 10 M urea.

Table 2. Solubility of Ni118 Precipitates

Solvent	Temperature	Time	Fraction dissolved
Miller-Golder-Buffer pH 6, I = 0,1	4° C	24 h	<0,05
Miller-Golder-Buffer pH 5, I = 0,1	30° C	24 h	<0,05
Miller-Golder-Buffer pH 10,5, I = 0,1	4° C	24 h	0,16
0,02 M Na-Dodecylsulfat pH 6, I = 0,12	20° C	<5 min	1,0
67% ~~Essigsäure~~ acetic acid	20° C	<20 sec	1,0
8 M Urea pH 6, I = 0,1	20° C	<5 min	1,0

Table 3. Native state half-lives of tr and ts proteins as a function of the incubation pH.

STRAIN	pH 5,0	pH 6,0	pH 7,0	pH 8,0
vulgare (*tr*)	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$
Ni 103 (*ts*)	$>2{,}5\cdot10^3$	400	20	20
Ni 118 (*ts*)	$>2{,}5\cdot10^3$	30	2	1,5
Ni 1927 (*tr*)	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$
CP415 (*ts*)	$>2{,}5\cdot10^3$	200	6	2
flavum (*ts*)	$>2{,}5\cdot10^3$	200	10	10
A 14 (*tr*)	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$	$>2{,}5\cdot10^3$

Temperature 30°C, I = 01. Values given are the values of the $t_{1/2}$ (min). The native state criterion is solubility at pH 5.